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Identification of Bacteria that Contribute to Imp Degradation in Horse Mackerel

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Abstract

Inosinic acid (inosine monophosphate, or IMP) is a taste component of fish that is broken down by an IMP-degrading enzyme (IMPase), impacting flavor. To measure IMPase activity, the enzyme is extracted as a solution from homogenized fish flesh, the IMP is then degraded by the enzyme, and the production of phosphoric acid from IMP is quantified. However, the degradation of IMP by bacteria that can be present in fish muscle could potentially affect the quantitation of endogenous IMPase activity. In this study, we isolated two bacterial strains from the enzyme solution obtained from horse mackerel and investigated their ability to degrade IMP. The isolates were identified as *Pseudomonas fragi* and either *Pseudomonas veronii or Pseudomonas extremaustralis*, as determined by 16S rDNA analysis. Of the two isolates, only P. fragi was found to be able to degrade IMP. Furthermore, the influence of the bacteria on the detection of IMPase activity was only seen when the reaction time was extended beyond 24 h.

Keywords: Inosinic acid (IMP); IMPase; Bacteria; Enzyme activity; Enzyme reaction time

Introduction

Inosinic acid (IMP) is an important taste component of fish [1]. IMP is produced by the postmortem degradation of ATP as follows:

ATP→ADP→AMP→IMP→HxR→Hx

The degradation from ATP to IMP proceeds relatively early [2] and IMP accumulates in fish muscle. Because IMP is broken down into HxR (inosine) and Hx (hypoxanthine), which are non-taste components, by IMPase over time, the suppression of IMPase activity can help maintain taste [3]. It has been reported that there are multiple sources of IMPase [4] and the type and the quantity of IMPase vary with the type of fish. Therefore, the examination of multiple IMPases is necessary to determine suitable conditions for the maintenance of the taste component in fish varieties by the suppression of IMPase activity.

In general, IMPase (produced by the fish) is primarily responsible for IMP degradation. IMPase activity is typically measured following extraction from homogenized fish flesh as an enzyme solution. IMPase activity is determined by measuring the production of phosphoric acid when a finite quantity of IMP is added and the reaction is incubated under various conditions [3-9]. However, it has been reported that IMP can also be degraded by bacteria in Japanese oysters and chicken [10-12]. Thus, bacterial contamination of fish may influence the measurement of endogenous IMPase activity. We performed a study on the maintenance of IMP in horse mackerel muscle, and it was necessary to measure IMPase activity precisely. However, the possibility that IMP is degraded by a factor other than the endogenous IMPase was suggested. Bacteria are not normally present in fish meat muscle, thus bacterial contamination of the IMPase reaction is probably introduced via the experimental setup or the internal organs of the fish. Because bacteria grow exponentially, longer reaction times will result in the acceleration of the reaction rate as the bacterial population increases. This complicates the ability to measure IMPase activity definitively and reliably.

In this study, we examined an enzyme extract to confirm whether bacteria are present in fish muscle. Isolated bacteria were cultured and identified. Finally, we assessed the ability of the bacteria to degrade IMP, thus confirming that bacteria can affect the measurement of IMPase activity from fish. Furthermore, we determined the enzymatic reaction time to optimally measure IMPase activity without influence from bacterial contamination.

Materials and Methods

Sample preparation

Horse mackerel (Chiba-grown) were purchased at a retail store in Tokyo. IMPase was extracted from fish samples as follows. Horse mackerel dorsal meat was collected and homogenized in three volumes of ultra-pure water. The homogenate was dialyzed against ultra-pure water for 2 days, after which the dialysate was filtered (No. 1; Advantec Co., Ltd., Tokyo, Japan) and diluted twice at 10°C (enzyme solution). Aliquots were also filter-sterilized (0.2 μm; Advantec Co., Ltd., Tokyo, Japan) for additional analyses.

Assessment of IMPase activity and reaction time

IMPase activity was measured using a standard reaction mixture that consisted of buffer (28 mM maleic acid/ Tris/NaOH, pH 6.5), 1.6 mM IMP, and 12.5% of the enzyme solution, filter-sterilized enzyme solution, or ultra-pure water (as without enzyme) in a total volume of 4 mL. The reaction mixture was incubated at 20°C for 0–48 h, and the reaction was stopped at regular intervals by the addition of 10% perchloric acid (final concentration, 3.3%). The precipitate was separated by centrifugation at 13,040×*g* for 10 min, and the level of free phosphoric acid was determined using the molybdenum blue method [13]. The amount of phosphoric acid was thought to reflect the level of enzyme activity.

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Examination of the bacterial flora in the enzyme solution

The presence and number of bacteria in the enzyme solution was determined by standard plate count. An aliquot (0.1 mL) of the enzyme solution was spread on plates with a standard agar medium (Pearl Core; Eiken Chemical Co., LTD), cultured at 20°C for 48 h. The bacterial flora present in the enzyme solution consisted of two predominant and distinct colony types. These two bacteria were sub-cultured to purity and identified by sequencing the 16S rRNA gene. Sequencing was performed by TechnoSuruga Laboratory Co., Ltd. (16S r-DNA-500 service; Shizuoka, Japan). Cells were lysed with achromopeptidase (Wako Pure Chemical Industries, Ltd., Osaka, Japan) to obtain a DNA template. PrimeSTAR HS DNA Polymerase (Takara Bio Inc., Shiga, Japan) and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) were used for the sequencing reactions. Base sequence determination was accomplished with ChromasPro 1.7 (Technelysium Pty Ltd., Tewantin, AUS) and Aporon 2.0 (TechnoSuruga Laboratory Co., Ltd.) was used for sequence analysis. Aporon DB-BA9.0 (TechnoSuruga Laboratory Co., Ltd.) was used to make comparisons to the International Nucleotide Sequence Databases (GenBank/DDBJ/EMBL).

IMP degradation by bacteria isolated from the enzyme solution

The two bacterial flora that were isolated from the enzyme solution were sub-cultured in liquid medium (Standard I Nutrient Broth containing of 15 g Bacto peptone, 3 g Bacto malt extract, 6 g NaCl, and 1 g glucose in distilled water to 1 L; Merck Ltd, Japan [26]) at 20°C for 24 h. After separation by centrifugation (10,000×*g*, for 10 min), bacteria were washed and suspended in 1 mL of 0.9% saline (bacterial solution). A 0.1 mL aliquot of the bacterial solution was cultured at 20°C for 24 h. The bacterial solution was added to the filter-sterilized standard reaction mixture described in Assessment of IMPase activity and reaction time. The reaction mixture was incubated at 20°C for 0 to 48 h, and the reaction was stopped at 0 h, 24 h, 48 h by the addition of 10% perchloric acid (final concentration, 3.3%). The level of free phosphoric acid was determined using the molybdenum blue method [13]. At the same time, 0.1 mL of the bacteria solution was plated on standard agar medium, incubated at 35°C for 24 h, and the CFUs were counted.

Statistical analysis

Data from the level of free phosphoric acid were subjected to one-way analysis of variance using the least significant difference method ($p<0.05$). Data from regression lines were subjected to analysis of covariance using the least significant difference method ($p<0.05$).

Results

Assessment of IMPase activity in horse mackerel over time

To quantify the level of IMPase activity in horse mackerel, the production of phosphoric acid was measured over time (Figure 1). IMPase activity levels of 0.54 mg/L and 19 mg/L were observed at 3 h and 48 h, respectively, revealing that IMPase activity accelerated as the reaction time lengthened ($p<0.05$). We presented an approximate expression to show the relationship of enzyme activity and reaction time as a multinomial expression (a quadratic equation) and confirmed the correlation. The multinomial expression and correlation was $y=0.0068x^2 + 0.071x + 0.71$, $R^2=0.994$. High correlation was confirmed in a quadratic equation.

To determine if bacteria present in the enzyme solution contributed to the detected IMPase activity, filter-sterilized aliquots of the enzyme solution were tested alongside unfiltered aliquots (Figure 2). In addition, a negative control without the enzyme solution was included to determine if any IMP degradation was due to bacterial contamination during the assay setup. Here, the production of phosphoric acid was detected in both the filter-sterilized and unfiltered enzyme solutions over time ($p<0.05$); however, the amount of phosphoric acid did not increase in the negative control. Therefore, IMP degradation was not due to contamination during the assay setup. In addition, the approximate expression and correlation was $y=0.0068x^2 + 0.071x + 0.071$, $R^2=0.9994$. When unfiltered enzyme solution was used, the reaction rate was found to increase over time. However, when the enzyme solution was filter-sterilized prior to the reaction, the reaction rate remained constant and had a tendency to reach saturation by 24 h. This suggests that IMP was degraded by an additional enzyme activity, which was probably produced by bacteria present in the unfiltered enzyme solution.

Isolation and identification of the bacterial flora present in the enzyme solution

To determine the presence of bacteria in the enzyme solution, an aliquot was plated on a standard agar medium. After incubation, we confirmed the presence of bacteria. On the basis of colony morphology, there appeared to be two primary bacterial species present, designated HS-A and HS-B. Next, we identified the bacteria species based on the 16 rRNA gene sequence (Figures 3 and 4). HS-A was accordingly found to be most closely related to *Pseudomonas fragi* (percentage identity of 100%) and HS-B was most closely related to either *Pseudomonas veronii* (100%) or *Pseudomonas extremaustralis* (100%).

Degradation of IMP by the isolated bacteria

To determine if the isolated bacteria were able to degrade IMP, suspensions of pure cultures were used in the IMPase assay. When we counted a number of bacteria in each bacteria solution, the number of standard plate count bacteria colonies confirmed on the standard agar medium showed 2.84×10^9 CFU/mL (HS-A) and 6.99×10^8 CFU/mL (HS-B). We were able to confirm the existence of bacteria in each bacterial solution. Suspensions of HS-A (2.84×10^9 CFU/mL) or HS-B (6.99×10^8 CFU/mL) were added to the filter-sterilized reaction mixture and the ability on each bacteria to degrade IMP was examined (Table 1). The production of phosphoric acid by HS-A was detected (101 mg/L at 24 h and 139 mg/L at 48 h; $p<0.05$), indicating that it possesses some IMPase activity. Thus, it was found that IMP was degraded by HS-A over time. In contrast, the production of phosphoric acid was not detected by HS-B, indicating that it was not able to degrade IMP.

Examination of the enzymatic reaction time on the IMPase activity measurement

Since the presence of bacteria in the enzyme solution results in an acceleration of the reaction rate over time, it is important to limit the reaction time. It is necessary that we let IMPase degrade IMP until the product is detectable but cease the reaction before the bacterial influence is observed. Therefore, from Figure 2, we evaluated an approximate straight line until each time using a covariance analysis and we examined the enzymatic reaction time on the measurement of IMPase activity (Table 2). No significant difference was found by the slope and intercept on the linear approximate equation of the enzymatic reaction by using each filter-sterilized enzyme and unfiltered enzyme until 24 h. However, a significant difference was found at 39 h and 48 h.

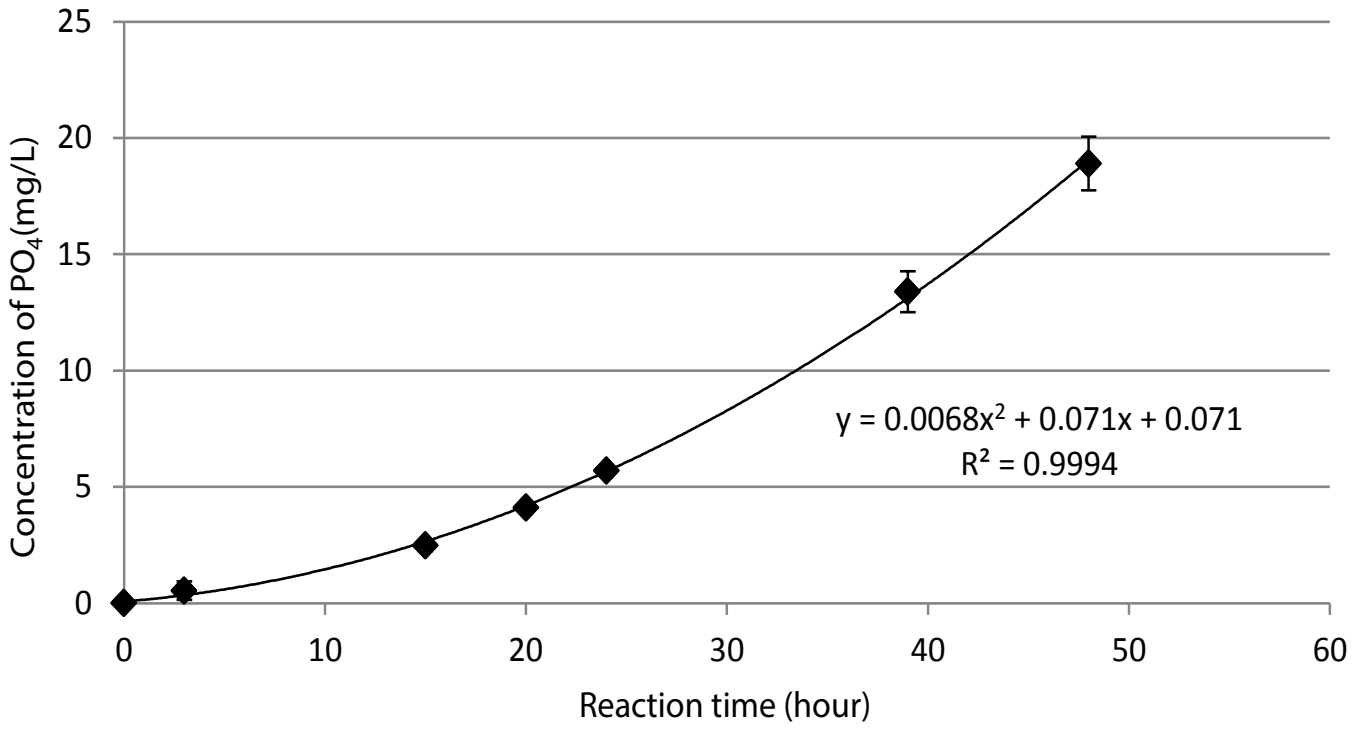


Figure 1: Change in the IMPase activity from horse mackerel over time (bars denote standard deviation of the mean; n=3). Mean values of relative activity indicate a significant ($p<0.05$) difference at each hour using a one-way analysis of variance.

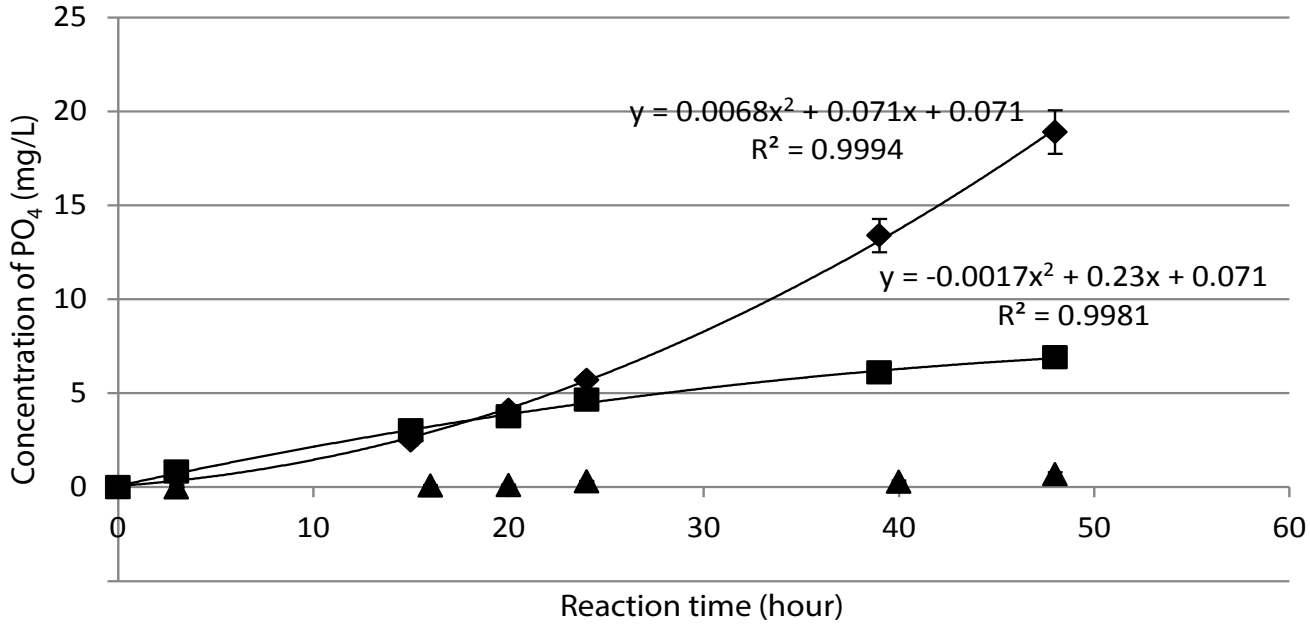


Figure 2: Changes in the IMPase activity of filter-sterilized enzyme solution (squares), unfiltered enzyme solution (diamonds), or negative control (triangles) over time (bars denote standard deviation of the mean; n=3). Mean values of relative activity indicate a significant ($p<0.05$) difference at each hour. The enzyme solution contained 1.4×102 CFU/mL at 24 h and 4.2×102 CFU/mL at 48 h.

Inoculated strain	Bacteria Count (CFU/mL)	Concentration of PO_4 (mg/L)	
		24h	48h
HS-A (*Pseudomonas fragi*)	2.84×10^9	101 (3.8)	139 (0.66)
HS-B (*Pseudomonas veronii* or *Pseudomonas extremaustralis*)	6.99×10^8	0	0

Table 1: Change in the amount of PO_4 and bacterial count of reaction mixture. Mean value of three independent determinations (n=3). Standard deviations are indicated in parentheses. Mean values of the amount of PO_4 indicate a significant ($p<0.05$) difference between 24 h and 48 h for HS-A.

Time	linear approximate equation		P	
	Not filtered	Filtered	Slope	Intercept
0-24h	y=0.22x-0.21 (0.96)	y=0.18x+0.13 (1.0)	0.207*	0.684*
0-39h	y=0.33x-1.2 (0.92)	y=0.16x+0.39 (0.97)	0.00868**	—***
0-48h	y=0.39x-1.9 (0.94)	y=0.14x+0.57 (0.97)	0.000289**	—***

Table 2: The comparison of linear approximate equation on the enzymatic reaction until each time () shows coefficient of correlation, * $p>0.05$; not significant, ** $p<0.05$; significant, *** When significant difference was not confirmed in slope, the intercept was not examined.

Discussion

The degradation of IMP due to bacteria

An acceleration of the reaction rate was observed when the activity of IMPase was measured in extracts of horse mackerel, indicating the presence of an additional factor that contributes to IMP degradation. Typically, an enzymatic reaction will progress in a linear manner with a constant reaction rate. As the substrate is depleted, the reaction rate decreases [14]. Therefore, if it is only an enzymatic reaction, the reaction rate does not increase exponentially. Such an increase is a tendency that is generally observed in bacteria [15,16], and thus it is likely that bacteria present in the reaction may also degrade IMP.

Previously, the degradation of IMP due to bacteria was demonstrated when the addition of loramphenicol (CP), an antimicrobial, prevented the acceleration of the reaction rate of ATP degradation in oyster [11,12] or prawn [17]. ATP degradation was increasingly rapid in the absence of CP compared with the presence of CP after 4 days on oysters or prawns. In addition, the production of HxR and Hx decreased when cod mince was sterilized [18]. On the basis of these reports, it was suggested that IMP was degraded by bacteria but the source of the bacteria-either bacteria present in the muscles or contamination during assay setup-was not confirmed.

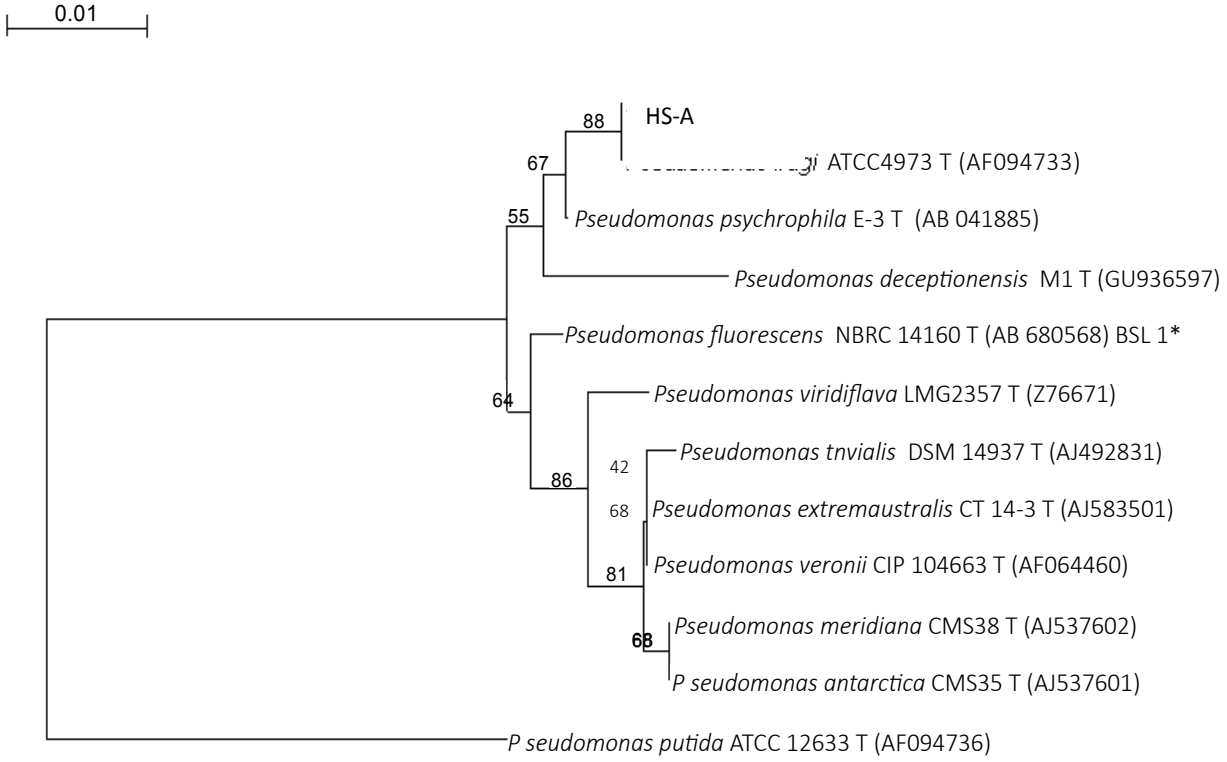
In this study, the degradation of IMP was attributed to the presence of bacteria in the enzyme extract from horse mackerel, not contamination during assay setup (Figure 2), thus revealing that bacteria are present in fish muscle. Although it is believed that the bacteria do not exist in the muscle of fish originally, it has been reported that bacteria can be introduced by physical factors such as the friction between fish in the fish catch, transportation, and catch landing [19]. In addition, it is possible that bacteria invade fish muscle from the internal organs and skin after death. When we investigated the varieties of bacteria present in horse mackerel, we found a number of *Vibrio* spp. and *Pseudomonas* spp. [20]. *Pseudomonas* spp. were identified among the bacterial flora that were extracted with the enzyme solution in this study (Figures 3 and 4). While the production of inosine nucleosidase by *Psuedomonas* spp. has been reported previously [21], there are no reports regarding the degradation of IMP by *Psuedomonas* spp.

The results of the phylogenetic analysis revealed that HS-A was most likely *P. fragi* and HS-B was either *P. veronii* or *P. extremaustralis*. Furthermore, it was found that HS-A had the ability to degrade IMP but HS-B did not (Table 1). It is possible that PO_4 was not detected when HS-B was added because of the difference in the bacterial numbers between reactions containing HS-A and HS-B. However, the IMPase activity assay was sensitive to PO_4 concentrations as low as 1 mg/L, the detection limit of this method. If HS-B had the ability to degrade IMP, IMP would have been degraded and PO_4 would likely have been detected. Thus, we concluded that HS-B is unable to degrade IMP. As shown in Figure 2, when an unfiltered enzyme solution was used, the amount of PO_4 at 48 h was 3–4 times that at 24 h. However, the amount of PO_4 at 24 h and 48 h were mostly the same as shown in Table 1. The amount of PO_4 shown in Figure 2 was lower than that shown in Table 1 because of the difference in the bacterial count. To confirm whether the bacteria degraded IMP, we used cultures that were sufficiently grown. Therefore, we considered that, because the amount of IMP was low relative to the bacterial count, the degradation of IMP at 48 h was saturated (Table 1). Confirmation that the upward deflection seen in Figure 2 is indeed attributable to bacterial activity will be necessary in the future.

P. fragi is one of the main bacterial flora in cooled and refrigerated edible meat and there are reports that *P. fragi* degrades protein in chicken [22], rabbit [23], and beef [24]. Accordingly, a protease that is responsible for hydrolyzing peptide bonds was isolated from *P. fragi* [24]. It has been reported that myosin was hydrolyzed by an enzyme isolated from *P. fragi* [23]. Previously, a bacterial protease has also been shown to have IMP degradation activity. The amount of free amino acid increased but the amount of sodium inosinate decreased when a bacterial protease was added to dried bonito [25,26]. Therefore, it is thought that IMP degradation by *P. fragi* was due to a protease.

Examination of the enzymatic reaction time on the IMPase activity measurement

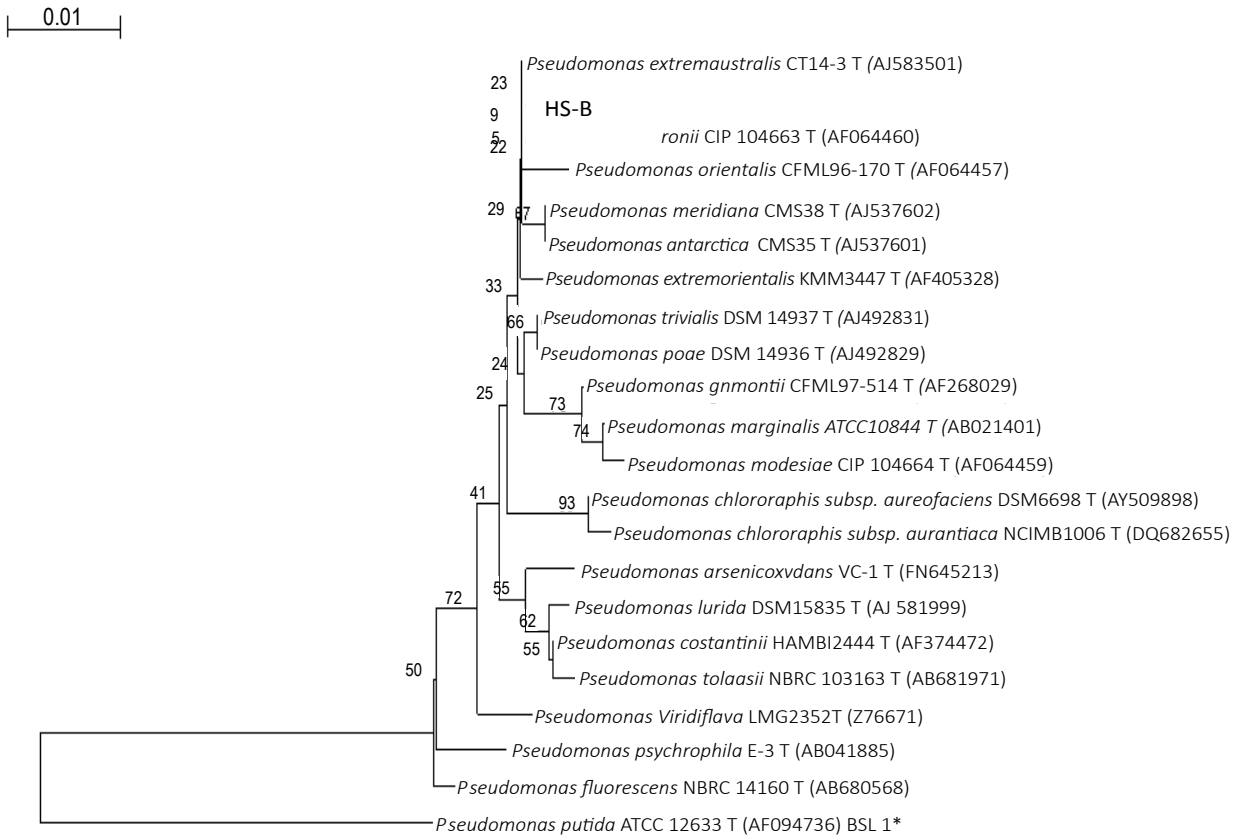
As no significant difference was found by the slope and intercept on the linear approximate equation of the enzymatic reaction using filter-sterilized and unfiltered enzyme solution until 24 h, we can disregard the bacterial influence for time points prior to 24 h. However, as a significant difference was found at 39 h and 48 h, it was concluded that bacterial growth influenced the measurement of IMP degradation at longer incubation times. Therefore, sterilization of the enzyme solution is not required by the time that IMPase activity is detectable but it is desirable to stop the reaction by 24 h. In addition, a tendency to be saturated 24 h later was seen in the enzymatic reaction with the



DNA base sequence on HS-A

GAGTTTGATCCTGGCTCAG
ATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGTAGAGAGAAG
CTTGCTTCTCTTGAGAGCGGCGGACGGGTGAGTAATACCTAGGAATCTGCCTGA
TAGTGGGGGATAACGTTCGGAAACGGACGCTAATACCGCATACGTCCTACGGGA
GAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTA
GCTAGTTGGTGAGGTAATGGCTCACCAAGGCTACGATCCGTAACTGGTCTGAGA
GGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCA
GCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTG
TGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCATT
AACCTAATACGTTGGTGTCTTGACGTTACCGACAGAATAAGCACCGGCTAACTCT
GTGC
CAGCAGCCGCGGTAATAC

Figure 3: Phylogenetic tree based on the 16s rRNA gene sequence of HS-A. Horizontal bars denote unit distance. The scale bar is indicated as a line in the lower left. Bootstrap values (%) are shown on the internal branches. “T” of the end of the strain name indicates a type strain.



DNA base sequence on HS-B

GAGTTTGATCCTGGCTCAG
ATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGTAGAGAGAAG
CTTGCTTCTCTTGAGAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTG
GTAGTGGGGGATAACGTTCGGAAACGGACGCTAATACCGCATACGTCCTACGGG
AGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATT
AGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGA
GAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGG
CAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCG
TGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCA
GTTACCTAATACGTGATTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACT
CTGTGC
CAGCAGCCGCGGTAATAC

Figure 4: Phylogenetic tree based on the 16s rRNA gene sequence of HS-B. Horizontal bars denote unit distance. The scale bar is indicated as a line in the lower left. Bootstrap values (%) are shown on the internal branches. "T" of the end of the strain name indicates a type strain.

filter-sterilized enzyme solution. When the reaction time was extended past 24 h, it is necessary to sterilize the enzyme solution. In the present study, we examined IMPase activity in only a single species, the horse mackerel, and therefore, in the future, it will be necessary to investigate IMPase activity in other fish species.

Conclusion

In this study, we isolated, cultured, and identified bacteria from fish muscle. We assessed the ability of the bacteria to degrade IMP and determined the enzymatic reaction time to optimally measure IMPase activity in the absence of any significant influence from bacterial contamination. *Pseudomonas fragi* and *Pseudomonas veronii* or *Pseudomonas extremaustralis* were isolated from horse mackerel and *Pseudomonas fragi* was able to degrade IMP. In addition, no significant difference was found on the linear approximate equation of the enzymatic reaction using filter-sterilized and unfiltered enzyme solution until 24 h. For the future analysis of IMPase activity, the use of a filter-sterilized enzyme solution would be advantageous to eliminate the possibility of confounding bacterial contamination. Furthermore, the activity becomes more consistent and robust when using a filter-sterilized enzyme solution for 24 h. However, we need to investigate the relationship between enzyme activity and reaction time because IMP degradation may become saturated when unfiltered enzyme solution is used. The measurement of IMPase activity is difficult because it can be affected by various factors. In addition to the method described here, there is a measurement method that uses an independently defined IMPase enzyme of known concentration, and also protein assays. These will possibly lead to future improvements in the measurement method.

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